

BMN673 sensitizes rhabdomyosarcoma tumors to irradiation *in vivo*

Research Thesis

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**Abstract**

Bone and soft tissue sarcomas are mesenchymal tumors that occur rarely in adults, representing only 1% of total malignancies, but comprise up to 13% of malignant tumors in children.<sup>1</sup> Rhabdomyosarcoma, a soft tissue sarcoma that commonly affects children, and osteosarcoma, a common bone sarcoma, exhibit aggressive tendency to metastasize and are associated with poor prognosis, high recurrence, and treatment failure.<sup>1</sup> Sarcoma, as well as other forms of cancer, can be treated with chemotherapeutic drugs that inhibit the actions of the poly (ADP-ribose) polymerase enzyme family, which catalyze the transfer of ADP-ribose to proteins and contribute to the repair of single-stranded DNA breaks.<sup>2</sup> Because some sarcoma cell lines display reduced DNA repair activity, these tumors might be relying on the PARP pathway for regular repair and maintenance of DNA during division.<sup>3</sup> Because of this, PARP inhibition is targeted by molecules such as BMN673 (talazoparib), which has shown success as a treatment for BRCA1/2 and PTEN-deficient cell lines.<sup>2,4</sup> BMN673, a recently developed PARP inhibitor with excellent *in vitro* activity, has been shown to increase tumor radiation sensitivity to a far greater extent than other PARP inhibitors; this action has been demonstrated to reduce tumor progression *in vitro* and shows promise as a treatment strategy in the clinic.<sup>2</sup> Our study shows that the combination of BMN673 with radiation therapy reduces final rhabdomyosarcoma tumor size and slows tumor progression in mice.

## Introduction

Sarcoma is relatively rare in the general population; however, sarcomas comprise approximately 13% of childhood tumors, and they commonly affect children less than five years old.<sup>1</sup> Sarcomas, which are derived from primitive mesenchymal cells, are grouped into two classifications: bone and soft tissue.<sup>5</sup> Sarcomas are heterogenous in clinical presentation and in molecular characterization because tumorigenesis might occur at different stages of mesenchymal differentiation.<sup>6</sup> Primary bone sarcomas, other than parosteal osteosarcoma, disproportionately affect males. Osteosarcoma and Ewing sarcoma develop largely in individuals under fifteen years of age who are experiencing periods of rapid long bone growth.<sup>6</sup> While bone sarcomas may occur at any location of any bone, they commonly present in areas with the greatest growth potential such as the metaphyseal region, in the case of osteosarcoma, or the diaphyseal region, in the case of Ewing's sarcoma.<sup>7</sup> Soft tissue sarcomas have a slight male predominance, and can develop in the joints, fat, nerves, dermis, blood vessels, and muscle; owing to this wide variety of potential origin, there are approximately 40 histologically different types of soft tissue sarcoma.<sup>5,8</sup> The focus of our study is on rhabdomyosarcoma, a soft tissue cell line, and osteosarcoma, a bone cell line, two of the most common types of sarcoma.

Clinicians frequently manage sarcoma with surgery and systemic chemotherapy; however, overall survival rates haven't improved beyond 70% in recent decades despite research and advancements in diagnostic and therapeutic technology.<sup>1,6</sup> Traditional chemotherapeutic agents commonly used to treat soft tissue sarcoma for the past several decades include doxorubicin (an anthracycline antibiotic that initiates dsDNA breaks by inhibiting topoisomerase), ifosfamide (an antineoplastic alkylator of DNA), gemcitabine (a deoxycytidine that inhibits DNA synthesis), and paclitaxel (an inhibitor of microtubule depolymerization during

mitosis).<sup>9</sup> Newer pharmacotherapies include olaratumab (an IgG monoclonal antibody that targets a growth receptor), eribulin (a microtubule inhibitor), and various poly (ADP-ribose) polymerase inhibitors such as BMN673, or talazoparib, the molecule that we investigated in this study.<sup>2,9</sup>

Inhibitors for poly (ADP-ribose) polymerase-1 (PARP1) and poly (ADP-ribose) polymerase-2 (PARP2), two important enzymes in the poly (ADP-ribose) polymerase (PARP) family, have been used with success in tumors that have deficiencies in the homologous recombination repair (HRR) pathway.<sup>2</sup> Tumors with this deficiency include common cancers of the breast and the ovaries with deleterious BRCA or PTEN deficiencies, which lead to the reduced ability to repair double-stranded breaks and decreased genomic stability. It is extremely important for cells to maintain genomic integrity during the stages of cell division, a process during which thousands of errors are made during genetic duplication; to ensure this integrity, the HRR pathway and the non-homologous end-joining pathway (NHEJ), among others, initiate

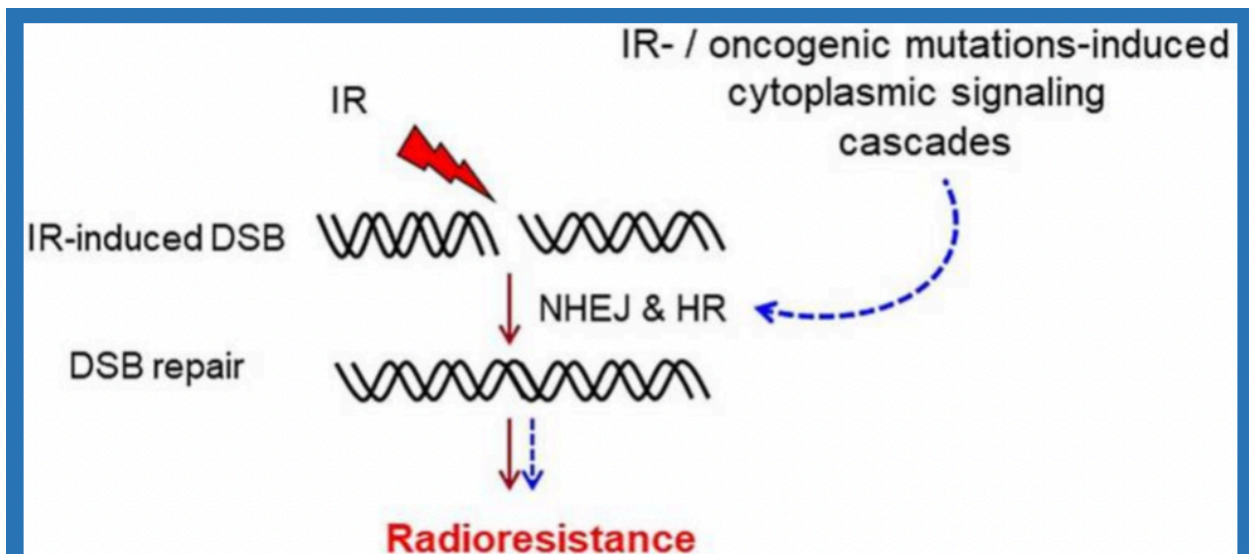


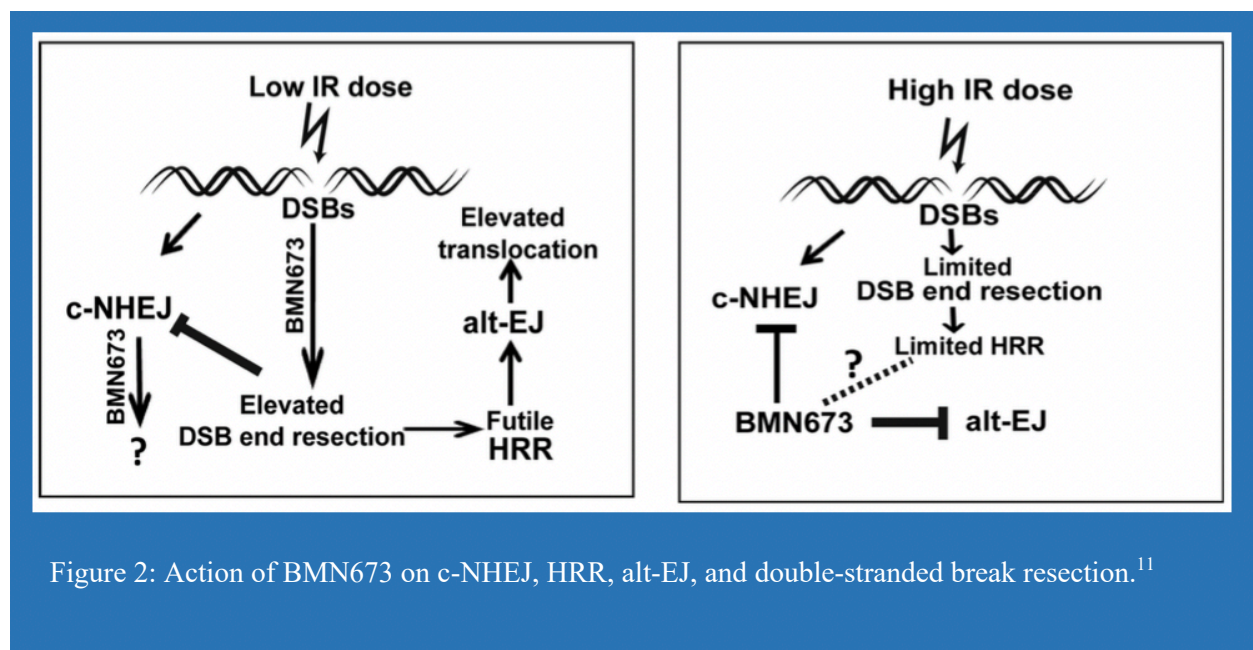
Figure 1: Mechanism of radioresistance in certain malignancies.<sup>11</sup>

repair of genetic damage caused by reactive oxygen species (ROS), UV light, and mutagenic chemicals.<sup>10</sup>

When common repair pathways for double-stranded breaks are defective, cells rely more heavily on the PARP pathway. In contrast to HRR's and NHEJ's functions in dsDNA breaks, the primary function of PARP is to identify single-stranded DNA (ssDNA) breaks and initiate the enzymatic response necessary to repair these single-stranded breaks.<sup>2</sup> Because cancerous cells with defective dsDNA repair pathways can utilize functional PARP to maintain genomic integrity, the PARP pathway is an excellent target for chemotherapeutic intervention. PARP1 may contribute to dsDNA break repair in HR deficient cells by promoting alternative end-joining (altEJ), an error-prone repair mechanism that functions when HRR and NHEJ fail to respond to damage. Notably, PARP1 is often overexpressed in HRR lacking cells.<sup>2</sup> Possibly for this reason, PARP inhibitors such as olaparib, veliparib, niraparib, and talazoparib (BMN673) are effective therapies in tumor cells lacking effective dsDNA repair mechanisms. In tumors with intact HR and NHEJ pathways, the role of PARP inhibition in successful treatment is not entirely understood. However, multimodal approaches to cancer treatment, including combining radiation therapy with chemical agents, show promise in this area: when BMN673 is combined with high doses of radiation, tumors become sensitized to radiation damage.<sup>2</sup>

Radiation therapy induces DNA double-stranded breaks, considered the most lethal form of genetic damage, in all cells but especially in malignancies with reduced genomic stability or rapid division.<sup>11</sup> Resistance to radiotherapy represents an obstacle to effective treatment. Exposure to radiation initiates a cellular response that includes activation of P13K/AKT, MAPK, STAT, and phospholipase C pathways that together increase the rate of cell division and post-radiation survival (Figure 1).<sup>11</sup> In tumor cells, this cascade can be

hyperactivated and leads to increased proliferation due to upregulated dsDNA break repair mechanisms like HRR and NHEJ.<sup>11</sup> While other PARP inhibitors exhibit only modest radiosensitization in tumor cells when combined with radiation therapy, BMN673 mediates strong radiosensitization and exhibits increased efficacy in damaging survival mechanisms in cancer by inhibiting PARP, NHEJ, and altEJ.<sup>11</sup> It also abrogates HRR and enhances double-stranded break end resection.<sup>2</sup> Compared to other PARP inhibitors, it displayed a short window of action (approximately 1 hour) and sensitizes tumors to radiation as effectively as NHEJ and HRR knockout models.<sup>2</sup> While olaparib generates sufficient radiosensitivity at 3 micromol/L, BMN673 is effective at concentrations as low as 10 nmol/L; furthermore, maximal radiosensitization is achieved *in vitro* with 50 nmol/L only one hour before irradiation in hamster CHO cells.<sup>2</sup> *In vitro* sarcoma experiments with BMN673 plus irradiation, dsDNA breaks were suppressed at relatively low radiation doses, irradiation-induced translocations were increased, and at high IR doses NHEJ and altEJ were more inhibited in comparison to other PARP inhibitors (Figure 2).<sup>11</sup> BMN673 *in vitro* sarcoma experiments suggest that it is a relatively low





impact and effective chemotherapy, and translational studies are needed to explore its effects in combination with radiation therapy.

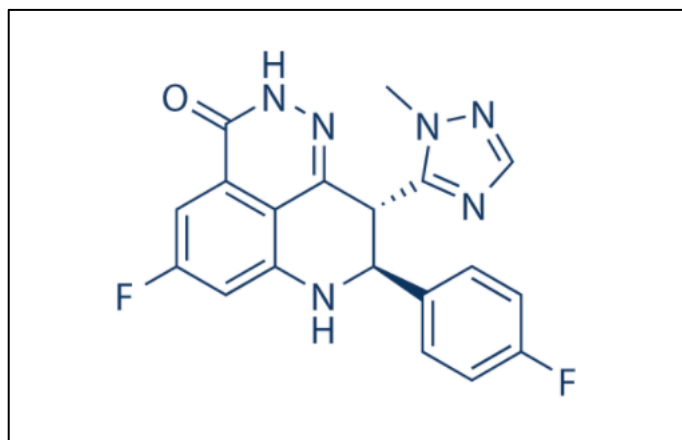


Figure 3: “BMN673.”<sup>12</sup>

	PARP1 enzyme inhibition IC <sub>50</sub> , nmol/L	Cellular PAR synthesis EC <sub>50</sub> , nmol/L	Capan-1 cytotoxicity IC <sub>50</sub> , nmol/L	Temozolomide potentiation GI <sub>50</sub> , nmol/L
Veliparib	4.73	5.9	>10,000	6,203
Rucaparib	1.98	4.7	609	144
Olaparib	1.94	3.6	259	237
LT-00628	1.82	4.5	8	5
BMN 673	0.57	2.5	5	3

Figure 4: “Summary of BMN673 *in vitro* activity.”<sup>13</sup>

## Methods

### I. *Cell culture*

All cell lines were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% air.

Rhabdomyosarcoma cell lines BVM02R and BVM05R and osteosarcoma cell line BVM3O were obtained from the oncology research labs of Washington University in St. Louis, Missouri which derived these lines from mouse sarcoma cells. All cell lines were grown in Dulbecco's modified version of Eagle's Minimal Essential Medium (DMEM) supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% amino acid solution. Cell lines were passed approximately three times per week.

### II. *Mice*

Three orders of thirty wild-type young female C57BL/6 mice (n = 90) were obtained from the Jackson Laboratory of Bay Harbor, Maine. Mice were pair-housed in climate-controlled suites in accordance with institutional guidelines and given access to food and water *ad libitum*. Each group of thirty mice received subcutaneous injection under anesthesia (2% isoflurane, 98% O<sub>2</sub>) of BVM05R, BVM02R, or BVM3O on the right flank behind the hip joint. Approximately three million cells were injected into each mouse for each cell line. In each group, mice were randomly assigned to one of four treatment cohorts: control, BMN673 alone, radiation therapy alone, or BMN673 combined with radiation therapy. All mice received oral gavage with BMN673 groups receiving 0.33 mg/kg drug daily prior to radiation therapy or sham radiation. Non-drug groups received vehicle via oral gavage. Tumors were measured beginning on the first day that tumors could be palpated with an area of approximately 2.5 mm x 2.5 mm. After this point, tumors were measured every other day until euthanasia due to tumor burden or at the end of 6-8 weeks. Tumor measurement was executed with a digital caliper. Mice were euthanized in

a CO<sub>2</sub> chamber in accordance with institutional guidelines when removal criteria were met concerning ulceration (2.0 x 2.0 mm), tumor size (greater than 1.6 x 1.6 cm), or general health concern (excessive anxiety-like symptoms indicated by hunched posture, weight loss, and reduced mobility).

### III. *Radiation*

Mice were subjected to sham radiation or radiation at the Ohio State University's College of Medicine Irradiation Core. The Radsource X-Ray Irradiator used is located in the vivarium of the Biological Research Tower on the medical campus. Mice in radiation treatment groups were subjected to four consecutive days of three gray (gy = J/kg) X-ray radiation and received anesthesia (2% isoflurane, 98% O<sub>2</sub>) prior to radiation.

### IV. *Statistical analysis*

Statistical analysis was conducted using UsableStats two-sample independent t-testing. Probability values were calculated between each data point using the final mean tumor volumes and final week standard deviations.

## Results

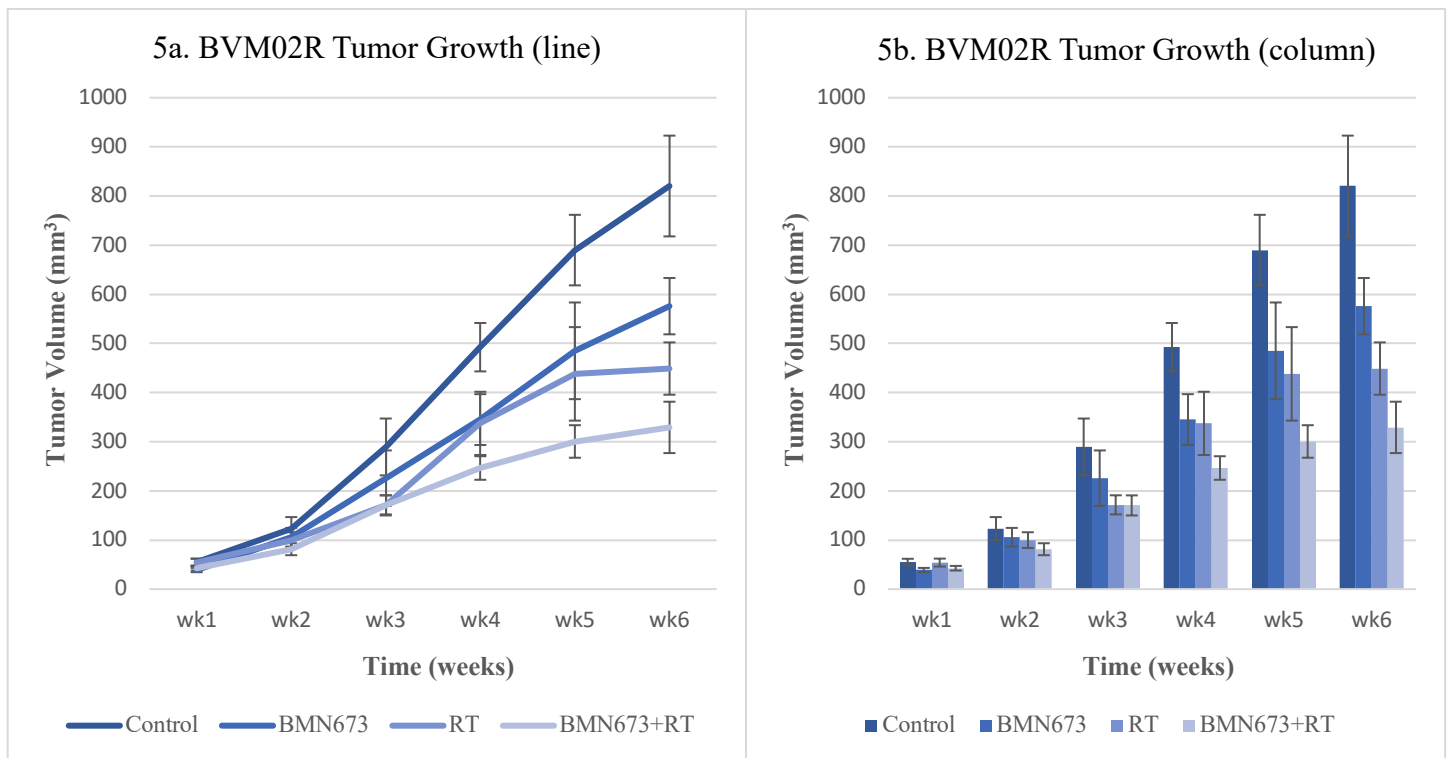
### I. *Observational results for mice injected with BVM02R, BVM05R, and BVM3O*

Mice injected with BVM02R and BVM05R rhabdomyosarcoma cells and BVM3O osteosarcoma cells exhibited rapid tumorigenesis and tumor growth over a period of six to eight weeks. In the BVM05R group, six mice developed exposed ulcerations on the middle portions of their tumor bodies due to necrosis. Mice with tumor ulcerations greater than 2.0 x 2.0 millimeters were euthanized via CO<sub>2</sub> chamber. Ulcerations typically developed in the center of the top portion of the tumor body. Tumors did not metastasize outside of the local area, and tumor size did not interfere with walking motion. In all groups, mice did not experience prolonged weight loss of greater than 10% and consumed food and water at normal rates. Mice with larger tumors, such as those in control groups, did not exhibit immobility or other anxiety-like symptoms. These mice responded to handling during tumor measurement similarly to other mice. There were no observed differences in behavior or activity between groups. Tumors with dimensions below 7.5 cm x 7.5 centimeters were symmetrical, while tumors with dimensions larger than 7.5 cm x 7.5 cm frequently exhibited elongation along the body's vertical axis. Larger tumors occasionally developed polyp-like growths on or near the base of the larger tumor.

### II. *Tumor measurements for BVM02R injected mice (Figure 5a, b; Table 1)*

The BVM02R-injected mice tumor measurement results were similar to the other rhabdomyosarcoma group (BVM05R). The experimental groups (BMN673, RT, and BMN673 + RT) all exhibited significantly inhibited tumor growth in comparison to control mice. The control group showed the fastest tumor growth over the course of the study. The BMN673 group that received sham radiation showed reduced tumor growth in each week compared to control (final

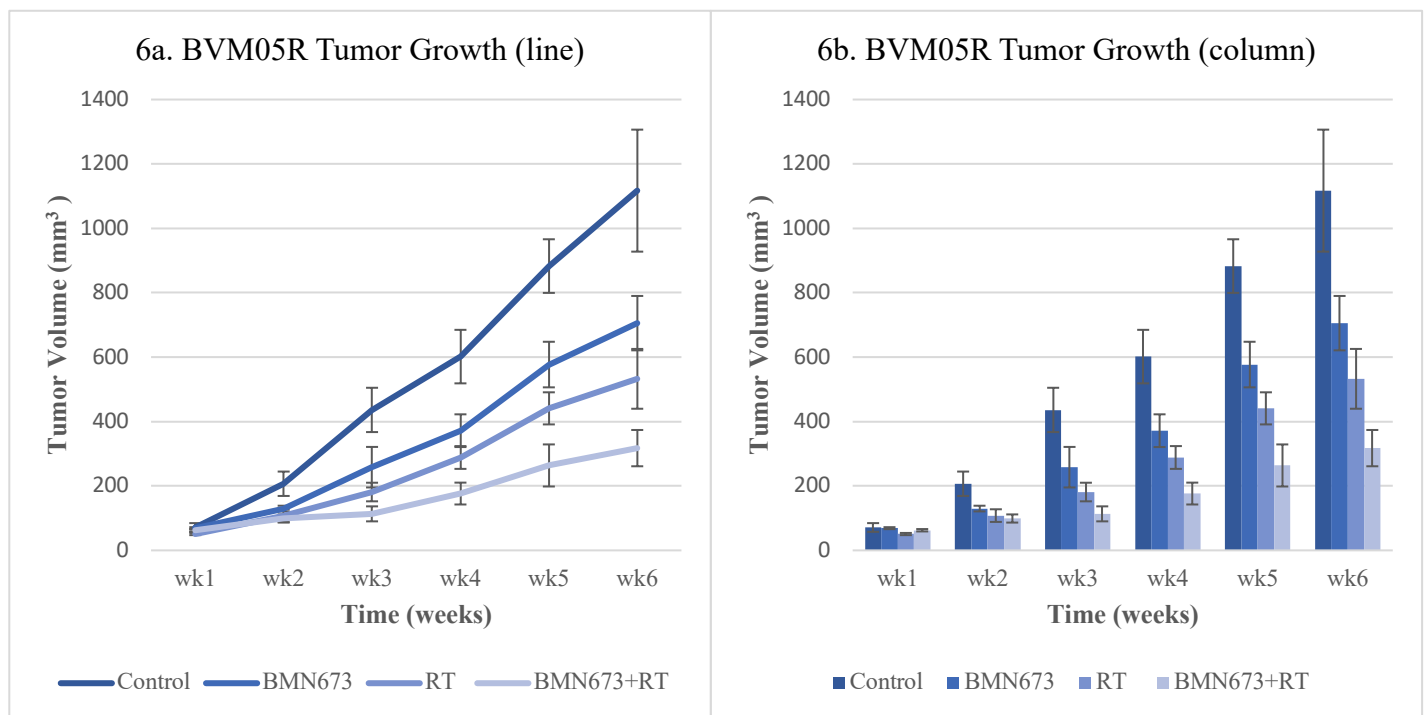
mean tumor volume difference =  $244.5 \text{ mm}^3$ ,  $p = 0.0112$ ). The radiation group that did not receive BMN673 exhibited reduced tumor growth in each week compared to BMN673 alone (final mean tumor volume difference =  $127 \text{ mm}^3$ ,  $p = 0.0274$ ). The BMN673 plus radiation group exhibited the slowest tumor growth and had the lowest mean tumor measurements in every week compared to the RT group (final mean tumor volume difference =  $119.7 \text{ mm}^3$ ,  $p = 0.0287$ ). These results show that BMN673 significantly sensitizes rhabdomyosarcoma cells to radiation therapy in the BVM02R tumor model.



**Figure 5:** BMN673 sensitizes BVM02R (rhabdomyosarcoma) sarcoma tumors to radiation therapy. From darkest to lightest coloring: Control, BMN673 alone, RT alone, BMN673+RT groups. BMN673 administered at 0.33mg/kg daily in BMN673-receiving mice. Radiation therapy administered at three gray over four consecutive days.

### III. Tumor measurements for BVM05R injected mice (Figure 6a, b; Table 1)

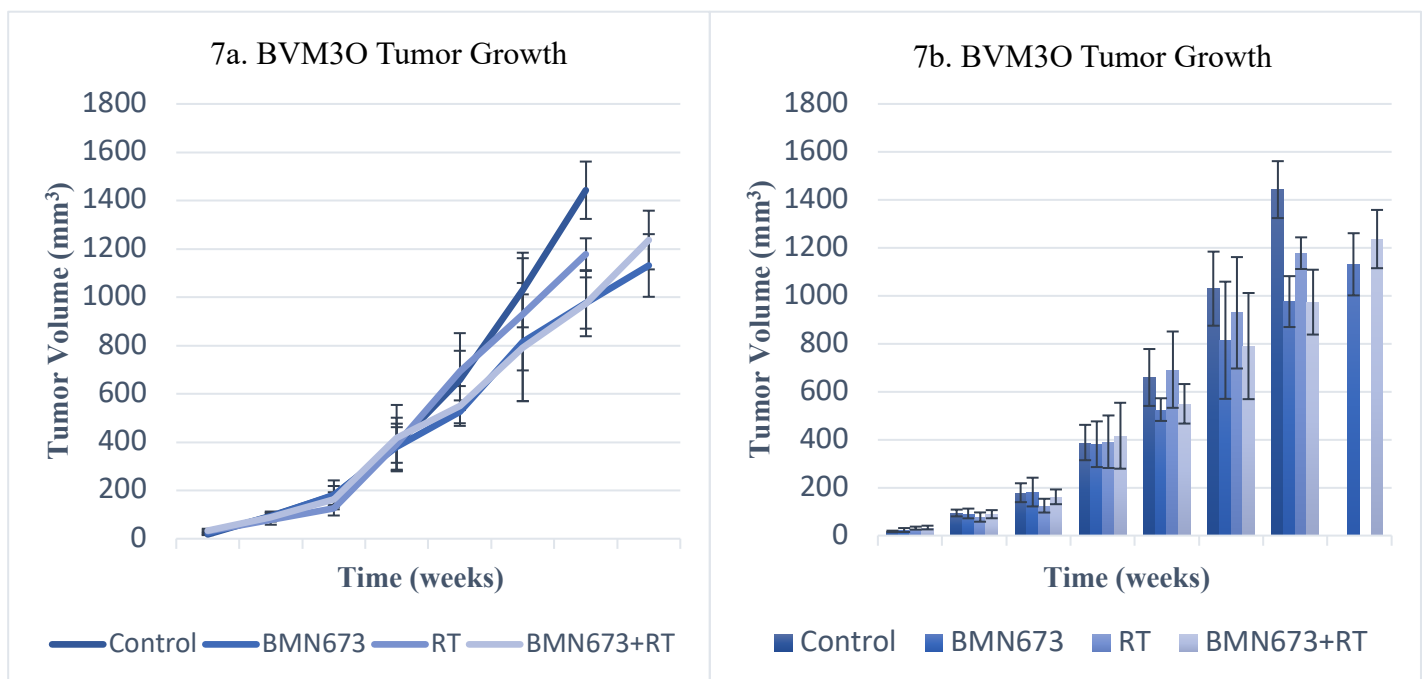
As mentioned above, the BVM05R tumor model experiment yielded similar results to that of the other rhabdomyosarcoma cell line, BVM02R. All treatment groups showed significantly reduced tumor volume in comparison to control at the end of the study, and BMN673 significantly increased sensitization to radiation therapy. The administration of BMN673 without radiation resulted in reduced tumor formation compared to the control (final mean tumor volume difference =  $411.5 \text{ mm}^3$ ,  $p = 0.0152$ ). This indicates, again, that the use of BMN673 as a chemotherapeutic agent in rhabdomyosarcoma is effective even without the combination of radiation therapy. The RT group exhibited even more inhibited tumor growth compared to control (final mean tumor volume difference =  $584.4 \text{ mm}^3$ ,  $p = 0.0032$ ), reinforcing the fact that radiation therapy alone was more effective than BMN673 alone. When these treatments were combined, the BMN673+RT group was even more effective than radiation alone (final mean tumor volume difference =  $215.1 \text{ mm}^3$ ,  $p = 0.0139$ ).



**Figure 6:** BMN673 sensitizes BVM05R (rhabdomyosarcoma) sarcoma tumors to radiation therapy. From darkest to lightest coloring: Control, BMN673 alone, RT alone, BMN673+RT groups. BMN673 administered at 0.33mg/kg daily in BMN673-receiving mice. Radiation therapy administered at three gray over four consecutive days.

#### IV. Tumor measurements for BVM3O injected mice (Figure 7a, b; Table 1)

BMN673 did not sensitize the BVM3O osteosarcoma tumor to radiation therapy. While all treatment groups were significantly more effective at reducing final tumor volume than control, the BMN673+RT group tumor measurements were almost exactly the same as those of the BMN673 group (final mean tumor volume difference =  $1.7 \text{ mm}^3$ ,  $p = 0.4949$ ). The least effective treatment was radiation therapy alone; however, radiation therapy still caused significant inhibition of tumor growth compared to control (final mean tumor volume difference =  $264.5 \text{ mm}^3$ ,  $p = 0.0162$ ). These results indicate that the most effective treatment in treating osteosarcoma was BMN673; the combination of this drug with radiation did not result in reduced tumor progression, and radiation therapy alone was less effective than BMN73 alone.



**Figure 7:** BMN673 does not sensitize BVM3O (rhabdomyosarcoma) sarcoma tumors to radiation therapy. From darkest to lightest coloring: Control, BMN673 alone, RT alone, BMN673+RT groups. BMN673 administered at 0.33mg/kg daily in BMN673-receiving mice. Radiation therapy administered at three gray over four consecutive days.

<b>BVM02R</b>	<b>Control</b>	<b>BMN673</b>	<b>RT</b>	<b>BMN673 + RT</b>
<b>Control</b>		$p = 0.0112$	$p = 0.0011$	$p = 0.0002$
<b>BMN673</b>	244.5		$p = 0.0274$	$p = 0.0007$
<b>RT</b>	371.5	127		$p = 0.0287$
<b>BMN673+RT</b>	491.2	246.7	119.7	
<b>BVM05R</b>	<b>Control</b>	<b>BMN673</b>	<b>RT</b>	<b>BMN673 + RT</b>
<b>Control</b>		$p = 0.0152$	$p = 0.0032$	$p = 0.0005$
<b>BMN673</b>	411.5		$p = 0.0478$	$p = 0.0002$
<b>RT</b>	584.4	172.9		$p = 0.0139$
<b>BMN673+RT</b>	799.6	388.1	215.1	
<b>BVM3O</b>	<b>Control</b>	<b>BMN673</b>	<b>RT</b>	<b>BMN673 + RT</b>
<b>Control</b>		$p = 0.0032$	$p = 0.0162$	$p = 0.0012$
<b>BMN673</b>	471.0		$p = 0.0560$	$p = 0.4949$
<b>RT</b>	264.5	206.5		$p = 0.0236$
<b>BMN673+RT</b>	472.7	1.7	208.2	

Table 1: Final mean tumor volume differences and  $p$  values for Control, BMN673, RT, and BMN673+RT in BVM02R, BVM05R, and BVM3O. Final mean tumor volume differences (blue) correspond to groups listed on top and on the left. Statistical analysis conducted using two-sample independent t-test. Probability values (orange) correspond to row and column groups.



## Discussion

The purpose of our study was to investigate the promising results of *in vitro* experiments of BMN673 in an animal model. Our investigation of BMN673's potential to sensitize sarcoma to irradiation *in vivo* confirmed the conclusions drawn from *in vitro* studies conducted by the lab of Dr. George Iliakis. The drug proved to be an effective intervention when used alone in models of rhabdomyosarcoma and osteosarcoma, and it proved to be even more effective as a combinational therapy when combined with x-ray radiation in rhabdomyosarcoma; however, this was not an effective combination in osteosarcoma treatment. The results we generated necessitate further exploration of the mechanisms underlying sensitization and resistance to radiation therapy.

We selected rhabdomyosarcoma as the primary focus of the study because it is one of the most common soft tissue sarcomas and responds to both chemotherapy and radiotherapy in humans. We selected osteosarcoma because it is susceptible to chemotherapy, but it is *not* very susceptible to radiotherapy; in fact, osteosarcoma is almost always treated with surgery and chemotherapy because it is notoriously resistant to RT.<sup>14</sup> This resistance is likely mediated by microRNA-driven upregulation of enzymes like human apurinic/apyrimidinic endonuclease 1 (APE1).<sup>15</sup> In light of our results, this response profile in comparison with that of rhabdomyosarcoma provides evidence that the observed effectiveness of the combined BMN673+RT treatment group was caused by a novel, synergistic mechanism rather than the additive effects of distinct mechanisms. Our results indicate that (1) the growth-inhibiting mechanism of BMN673 is not the same as that of radiotherapy, though the two may overlap, (2) that BMN673 has moderate activity as a singular therapy in both models, (3) that BMN673

increases the susceptibility of rhabdomyosarcoma to radiation therapy, and (4) that BMN673 does not potentiate radiation in osteosarcoma, a subtype of sarcoma that is resistant to radiation.

The mechanism of this synergistic effect remains unknown. We know that radiation causes the formation of double- and single-stranded breaks in DNA, and that BMN673 inhibits the action of PARP1. However, PARP1 primarily functions in the repair of single-stranded breaks; in rhabdomyosarcoma, the double-stranded repair mechanisms are likely intact. HRR and NHEJ may even be upregulated in response to irradiation. A possible explanation of the observed effect is that PARP1 is unable to repair single-stranded breaks caused by radiation, and when single-stranded breaks in DNA reach a replication fork, double-stranded breaks often form. The tumor cells' error-free repair pathways may be overwhelmed by genomic instability, and the cells may begin to partially rely on altEJ and other error-prone repair mechanisms to overcome this instability. However, in addition to PARP1 inhibition, BMN673 was shown *in vitro* to reduce the activity of secondary repair pathways like altEJ and primary pathways like HRR and NHEJ and, in addition, to enhance end resection of double stranded breaks. *In vitro*, the cumulative effect of these actions resulted in tumor responses to PARP inhibition seen only in HRR and NHEJ knockout models. It is likely that BMN673 exerted a similar battery of molecular mechanisms in the animals; however, this remains unknown. If BMN673 is inhibiting PARP, modulating HRR and NHEJ, reducing the activity of altEJ, and interfering with the resection of double-stranded breaks, it is a significant and promising intervention for sarcoma and for many other cancers as a singular therapy. Even more so, the combination of BMN673 with irradiation proved to be significantly more effective than either therapy alone, and further translational studies are needed to develop a better idea of the molecular mechanics and safety profile of this drug in combination with RT.

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